The effect of high frequency stimulation on intracellular Ca^{2+} in sympathetic PC12 cells☆

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Abstract

Objective. This study were designed to compare the impact of instant high frequency stimulation (HFS) and 3-day continuous HFS on the intracellular Ca^{2+} volume in sympathetic PC12 cells and to define the role of Nifedipine(NIF) in this process. *Methods.* The cells were randomly divided into two groups, namely instant HFS and 3-day continuous HFS. Using a line scanning technique under a Laser Scanning Microscope (LSM) at 1.2 ms – 1.25 ms intervals for 60s, we found that the intracellular Ca^{2+} fluorescence intensity changes between conduction of HFS and after termination of HFS. *Results.* The intracellular Ca^{2+} fluorescence intensity rapidly declined instantly after HFS on the sympathetic PC12 cells and the range of declination observed through line scanning was 47.7%. HFS was conducted continuously for 3 days, 3 hours per day. After discontinuing HFS, Ca^{2+} fluorescence intensity increased sharply with a 60% inclination. This effect was weakened by Ca^{2+} channel inhibitor Nifedipine (NIF). *Conclusion.* These data indicates that the variation of Ca^{2+} volume induced through HFS has a significant effect on sympathetic PC12 cells. On commencing instant HFS, Ca^{2+} fluorescence intensity increased significantly and decreased sharply after 3-day continuous HFS was terminated. However, NIF can partially counteract the inhibitory effect of HFS on PC12 cells, instead of complete blockage.. [Life Science Journal. 2009; 6(2): –] (ISSN: 1097 – 8135).

Keywords: PC12 cells; high frequency stimulation; Nifedipine; laser scanning microscope

1 Introduction

The treatment of Parkinson's disease (PD) is a difficult process that currently remains partially explored. Now, high frequency stimulation (HFS) of the subthalamic nucleus (STN) has become one of the main global surgical techniques in the treatment of PD[1,2]. In clinical conditions, HFS of STN neurons in PD is empirically applied at > or = 100 Hz (130 – 185 Hz), with pulses of short duration (60 – 100 micros) and 1 – 3 mA amplitude[3,4]. And more recently, a double effect hypothesis, implying both an inihibition of pathological neural activity and the induction of a new rhythm by periodic electrical stimulation[5,6]. This experiments attempts to enhance understanding of HFS effect mechanisms in treating PD and to observe changes in cellular function under stimulation more effectively, in particular Ca^{2+} as the second messenger which partakes widely in a large range of cellular functions such as cellular movement, secretion, metabolism and differentiation[7], as well as Ca^{2+} mediated cells also have significant tuning effects on responses to external stimulation[8]. This experiment utilizes pheochromocytoma cells (PC12 cells) that are similar to neurons in cellular anatomy, physiology and biochemistry, and possess common attributes with neurons and neurosecretory cells[9], when induced by nerve growth factor (NGF) in particular, the PC12 cell differentiates toward sympathetic neurocytes[10,11]. We applied HFS on continuous sympathetic PC12 cells by currents acting on medium, and using a laser scanning microscope (LSM) observed changes in sympathetic PC12 intracellular Ca^{2+} under the effect of HFS, and through the use of Nifedipine (NIF), prove that changes can be induced in sympathetic PC12 intracellular Ca^{2+} through HFS. We expect these results would benefit further exploration of possible effect mechanisms HFS has on the treatment of

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PD, and provide more reliable theoretical support for the appropriate use of HFS in the clinical treatment of PD.

2 Materials and Methods

2.1 High frequency stimulation method

Cells were inoculated in a Poly-L-Lysine hydrobromide (PLL) (Sigma, USA) coated 24-hole culture plate or a 3.5 cm culture dish and incubated for 24 hours in a 37 ºC incubator with 5% CO_2. Titanium wires were aligned parallel across adjacent holes in the 24 hole culture plate, two titanium wires were aligned parallel in the 3.5 cm culture dish, with ends respectively connected to positive and negative currents and stimulated with high frequency electrical currents. Square wave was generated by a stimulus generator and run through the isolator to stimulate cell population. HFS parameters were: 130 Hz frequency, 500 μA current, 60 μs pulse widths.

2.2 Incubation of sympathetic PC12 cells and cellular identification experiment

Undifferentiated PC12 cells were provided by the Nuclear Medicine Lab, College of Medicine, Shanghai Jiao Tong University. Incubation fluid contained 88% Dulbecco's modified Eagle's medium (DMEM) (GIBCO, USA), 10% fetal bovine serum (Zhejiang Hangzhou Sijiqing Biotech Co., China), 1% Penicillin streptomycin (GIBCO, USA) and 1% L-Glutamine (GIBCO, USA). PC12 cells were incubated in a 37˚C incubator with 5% CO_2; Cells were inoculated in a 24-hole culture plate at a density of 0.5×10^4 units/cm^2 or which were inoculated in a 3.5 cm culture dish at a density of 0.8×10^4 units /cm^2. 72 hours before commencing the experiment, sympathetic neurocytes obtained by inducing and differentiating PC12 cells through NGF (Promega, USA) were added at a final concentration of 50 ng/ml[11,12]. The cells were left overnight using 1 : 4000 TH-16 (Sigma, USA) at 4 ºC, with anti-mouse-HRP (Shanghai Changdao Biotech Co., China) incubation at 37 ºC for 1 hour to conduct immunohistochemical staining. The positive results of tyrosine hydroxylase (TH) which secreted by PC12 sympathetic neurocytes were yellow-brown particles.

2.3 Instant high frequency stimulation

2.3.1 Changes in intracellular Ca^{2+} in PC12 cells. Cells were inoculated at a density of 0.8×10^4 units/cm^2 in a PLL coated 3.5 cm culture dish containing 3 ml of fluid culture. NGF was added at a final concentration of 50 ng/ml to induce differentiation in PC12 cells. After cells have adhered, 0.5 mM of Fluo-4/AM (Molecular Probes, USA) with a final concentration of 2.5 μM was added to monitor PC12 intracellular Ca^{2+}. Cells were then incubated for 30 minutes in an incubator with 5% CO_2 at 37 ºC. The culture dish was placed under scanning stage of laser scanning microscope (510) (Zeiss Co., German) at × 10 objective and excited by an argon ion laser with excitation wavelength 488nm and emission wavelength BP 500-550 nm. Images were recorded via photomultiplier and image resolution was 52 × 52 using imaging software Time Series v.4.0. Line scanning was conducted at 1.2 ms – 1.25 ms intervals, with a total of 50000 scans over a total time of 60 seconds. HFS (parameters same as above) was conducted 14.4 seconds after commencing line scan and changing trends in intracellular Ca^{2+} fluorescence were dynamically recorded.

2.3.2 The effect of NIF on intracellular Ca^{2+} in PC12 cells. Cells are differentiated using NGF and identified by the TH-16 immunohistochemical method, then inoculated in a 3.5 cm culture dish at 0.8×10^4 units/cm^2. PC12 intracellular Ca^{2+} were measured with Fluo-4/AM and cells were incubated for 30 minutes in an incubator with 5% CO_2 at 37 ºC, then incubated with NIF (Calbiochem Co., USA) (final concentration 2 μM) for 5 minutes under the same conditions. Changes in intracellular Ca^{2+} fluorescence upon commencing HFS were observed under a LSM using the same scanning parameters as above.

2.4 Three-day continuous high frequency stimulation

2.4.1 Changes in intracellular Ca^{2+} in PC12 cells. Cells were differentiated through NGF and identified with the TH-16 immunohistochemical method, then inoculated in a 3.5 cm culture dish at 0.8×10^4 units/cm^2. After cells have adhered, HFS was applied continuously for 3 hours at fixed times daily for three days. HFS (parameters same as above). 30 minutes before terminating the 3-day stimulation period, PC12 intracellular calcium ions were measured with Fluo-4/AM and cells were incubated in an incubator with 5% CO_2 at 37 ºC, and scanned under a LSM at 1.2 ms – 1.25 ms intervals, with a total of 50000 scans over a total time of 60 seconds. HFS (parameters same as above) was conducted 14.4 seconds after commencing line scan and changing trends in intracellular Ca^{2+} fluorescence were dynamically recorded.

2.4.2 The effect of NIF on intracellular Ca^{2+} in PC12 cells. Cells were differentiated using NGF and identified by the TH-16 immunohistochemical method, then inoculated in a 3.5 cm culture dish at 0.8×10^4 units/cm^2. After cells have adhered, HFS (parameters as above) was applied continuously for 3 hours at fixed times daily for three days. 30 minutes before terminating the 3 day stimu-

lation period, PC12 intracellular Ca^{2+} were measured with Fluo-4/AM and cells were incubated for 30 minutes in an incubator with 5% CO_2 at 37 ºC, then incubated with NIF (final concentration 2 μM) for 5 minutes in an incubator with 5% CO_2 at 37 ºC, and scanned under a LSM (same scanning parameters as above) to observe effects of NIF on changes and trends in intracellular Ca^{2+} fluorescence values instantly after the 3-day HFS period was terminated.

2.5 Statistical analysis

Data drawn from cell form and cellular identification were analyzed using imaging software RS IMAGE PRO V.4.5; Laser scanned images were analyzed using imaging software Time Series. Data drawn from the experiment, in-group differences before and after HFS in the instant stimulation group and termination of the 3-day continuous stimulation group were tested using the one-way ANOVA. Differences between the instant stimulation group and 3-day continuous stimulation group were tested using the independent *t*-test and analyzed using SPSS 11.0.

3 Results

3.1 PC12 sympathetic neuron cell identification results

PC12 cells are differentiated by NGF to become sympathetic neurocytes. After HFS, the metabolic level of intracellular tyrosine hydroxylase is tested using the TH-16 immunohistochemical method. This method stains PC12 sympathetic neurocytes yellow-brown and serves to identify PC12 sympathetic neurocytes after HFS (Figure 1).

3.2 Changes in intracellular Ca^{2+} in PC12 cells after instant HFS

After Fluo-4/AM has been combined with free Ca^{2+} in PC12 sympathetic neurocytes, it could be observed using the LSM that intracellular fluorescence was evenly distributed. After commencing instant HFS, the strength of intracellular fluorescence weakened (Figure 2 A & B); it could be observed through line scanning that with the progression of stimulation time, intracellular Ca^{2+} fluorescence decreased sharply (Figure 3), with range of decline reaching 47.7% (Figure 4).

3.3 NIF on intracellular Ca^{2+} in PC12 cells after instant HFS

After adding NIF (2 μM), cells were incubated for 5 minutes before HFS. Change in intracellular fluorescence in the instant HFS group was not apparent before and after commencing HFS (Figure 5 A & B). It could be observed through line scanning method that intracellular fluorescence did not weaken significantly as stimulation time progressed (Figure 3), with range of only 4.8%, compared with groups without NIF ($P < 0.01$) (Figure 4). This indicated NIF had suppressed Ca^{2+} outflow induced by HFS.

3.4 Changes in intracellular Ca^{2+} in PC12 cells after 3-day continuous HFS

It could be observed that after HFS was terminated for the 3-day HFS group, there was a gradual increase in intracellular fluorescence (Figure 2 C & D). It could be observed through line scanning that after HFS was terminated, intracellular Ca^{2+} fluorescence increased rapidly (Figure 6) at range of 60% (Figure 7). Despite the speed and short time between line scan intervals that may cause fluorescence annihilation, line scanning still showed the more significant increase in Ca^{2+} fluorescence. After HFS was terminated for the 3-day continuous HFS group, intracellular Ca^{2+} fluorescence increased. This may have been caused by the inflow of extra-cellular Ca^{2+} or the release of intracellular calcium storage.

3.5 NIF on intracellular Ca^{2+} in PC12 cells after 3-day continuous HFS

After adding NIF (2 μM), cells of the 3-day HFS group were incubated for 5 minutes before HFS. Change in intracellular fluorescence was not apparent after HFS was terminated (Figure 5 C & D). It could be observed through line scanning method that intracellular fluorescence did not weaken significantly (Figure 6), with range of only 17.2% compared with the group without NIF ($P < 0.01$) (Figure 7). This indicated NIF had inhibited Ca^{2+} outflow induced by HFS in the 3-day HFS group, but from the data it is shown that NIF did not completely suppress changes that occurred in intracellular Ca^{2+}.

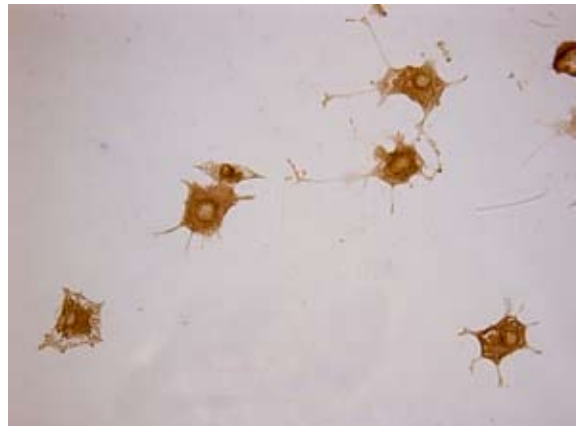

Figure 1. TH in PC12 sympathetic neurocytes was stained yellow-brown by TH-16 immunohistochemical method (DAB, × 400).

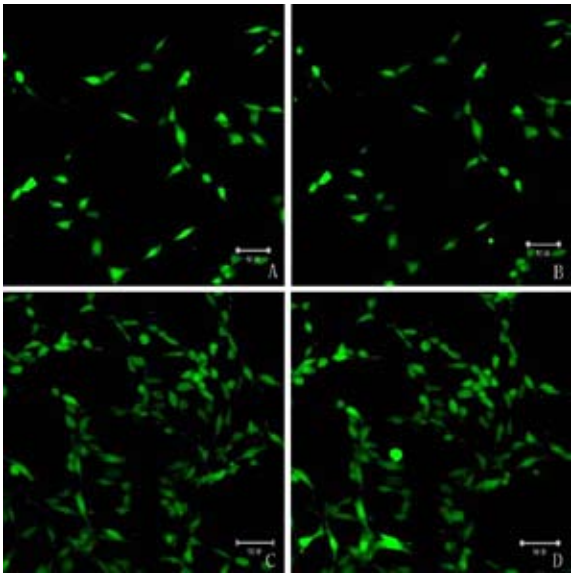

Figure 2. The effect of high frequency stimulation on intracellular Ca^{2+} in sympathetic PC12 cells. A: Before commencing instant HFS. B: After commencing instant HFS. C: Before terminating 3-day HFS. D: After terminating 3-day HFS.

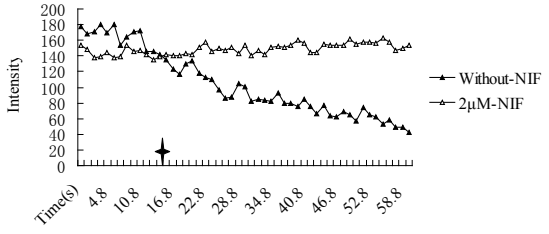

Figure 3. Line scanning of instant HFS. With commencing instant HFS, and without-NIF intracellular Ca^{2+} fluorescence decreased sharply; 2 μM-NIF intracellular Ca^{2+} fluorescence did not weaken significantly. †:Instant HFS was conducted 14.4 seconds.

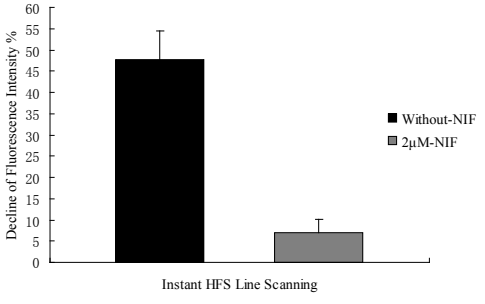

Figure 4. The effect of NIF on intracellular Ca^{2+} in PC12 cells after commencing instant HFS. With commencing instant HFS, and without-NIF intracellular Ca^{2+} fluorescence decreased sharply, with ranges of decline reaching 47.7% through line scanning;2μM-NIF intracellular fluorescence did not weaken significantly, with range of only 4.8%. **: $P < 0.01$ *vs.* the without-NIF group.

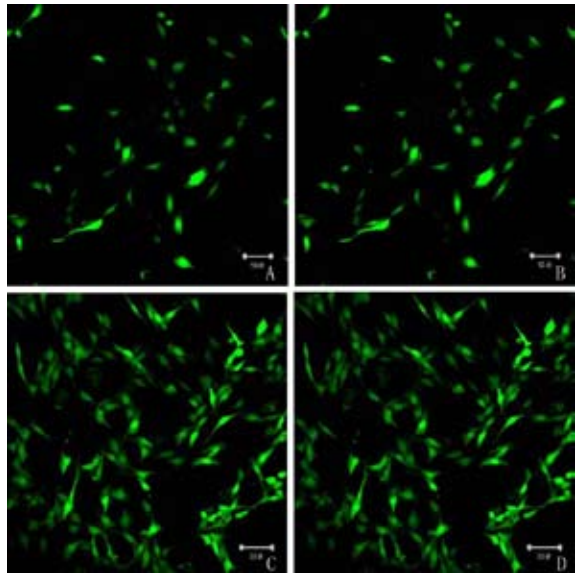

Figure 5. The effect of L-voltage sensitive Ca^{2+} channel blocker NIF on intracellular Ca^{2+} in PC12 sympathetic neurocytes after HFS. A: Before commencing instant HFS; B: After commencing instant HFS; C: Before terminating 3-day HFS; D: After terminating 3-day HFS.

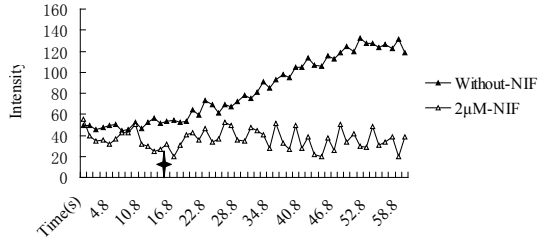

Figure 6. Line scanning of instant 3-day HFS.With terminating 3-day HFS, and without-NIF intracellular Ca^{2+} fluorescence increased rapidly;2μM-NIF intracellular Ca2+ fluorescence did not weaken significantly; :3-day HFS period was terminated 14.4 seonds.

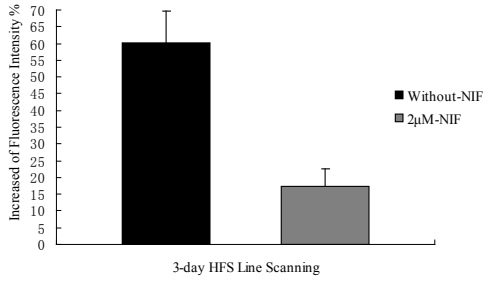

Figure 7. The effect of NIF on intracellular Ca^{2+} in PC12 cells after terminationg 3-day HFS. With terminating 3-day HFS, and without-NIF intracellular Ca^{2+} fluorescence increased rapidly at range of 60% through line scanning;2 μM-NIF intracellular fluorescence did not weaken significantly,with range of only 17.2% .**: $P < 0.01$ *vs.* the without-NIF group.

4 Discussion

Since TH is a rate-limiting enzyme organically composed by catecholamines as well as a key enzyme in the formation of such substances as dioxyphenylalanine and adnephrin, an important role in the organic synthetic tuning of substances such as dioxyphenylalanine. A range of abnormal changes generally occur in the genetic expression, apoenzyme levels and enzymatic activity of animal PD models and PD sufferers[13,14], and it was also found that TH levels increase significantly in PC12 cells that have been NGF differentiated[15]. The high frequency electrical currents act on cells' body by cells' medium[16]. Based on this, this experiment tests the metabolic level of intracellular TH using the TH-16 immunohistochemical method, which serves to determine whether NGF differentiated PC12 cells are sympathetic neurocytes and provides basis for successive experiments. The changes in intracellular Ca^{2+} fluorescence before and after commencing/terminating HFS was observed.

Intracellular Ca^{2+} plays the role of the second messenger; its temporary changes are determined by extracellular Ca^{2+} density and unpolarized cellular membrane level[17,18,19]. This experiment shows that there is a close relationship between HFS and intracellular Ca^{2+} fluorescence. Significant changes could be observed in intracellular Ca^{2+} fluorescence at the instant of commencing or terminating HFS. Upon commencing HFS, Ca^{2+} fluorescence increased significantly and decreased sharply after HFS was terminated. It is speculated that under stimulated conditions, the Ca^{2+} channel located on the PC12 cellular membrane is activated by the intra/extra-cellular voltage difference generated by instant HFS. This could cause the Ca^{2+} channel located on the PC12 cellular membrane to open, resulting in the rapid outflow of cytoplasm Ca^{2+}. Related research lends proof to the fact that electrical stimulation can cause changes in single calcium channel dynamics[20].

Through line scanning, this experiment showed that there is a regressive time-strength relationship between HFS and intracellular Ca^{2+} fluorescence. The decline rate of Ca^{2+} fluorescence which is caused by line scan is 47.7%. It is shown that instant HFS induces a stronger intracellular Ca^{2+} outflow for a short time after it commences, which subsides as time progresses. Upon termination of stimulation repeated daily at fixed times and frequencies for 3 days, Ca^{2+} located outside and in the cell organ may both be motivated to enter the cytoplasm. Not only extracellular free Ca^{2+} inflow but also the release of intracellular calcium storage cause localized calcium signals in Ca^{2+} in the cytoplasm to strengthen. Similarly, observations from line scanning techniques, it was shown that the increase rate of Ca^{2+} fluorescence caused by line scan is 60%. It can be seen that HFS also caused more obvious Ca^{2+} fluorescence signal in a short time and Ca^{2+} inflow subsides as time progresses after HFS has been terminated. Meanwhile, this effect is closely tied to the L-voltage sensitive Ca^{2+} channel, despite the fact that this channel plays a secondary role in the release of neurotransmitters[21]. The results of this experiment lend experimental evidence to the release of HFS induced intracellular Ca^{2+} via the L-voltage sensitive Ca^{2+} channel. When the channel inhibitor NIF is used in conjunction with HFS[22], it brings about significant changes in intracellular Ca^{2+} fluorescence, in which no apparent changes occurred at the instant commencing HFS and 3-day continuous terminating HFS. It is speculated that the Ca^{2+}channel inhibitor altered cell response toward different mediate signals. However, further research is needed to determine whether the increase and decrease of intracellular Ca^{2+} fluorescence upon commencing/terminating HFS are caused entirely by the opening of L-voltage sensitive calcium channels, and whether the strength of this effect is tied to NIF concentration.

5 Conclusion

This experiment shows that there is a close relationship between HFS and intracellular Ca^{2+} volume in PC12 cells. Significant changes in intracellular Ca^{2+} fluorescence intensity could be observed at the instant of commencing or terminating HFS. Upon commencing HFS, Ca2+ fluorescence intensity increased significantly while decreased sharply after HFS was terminated. And the NIF can partially counteract the inhibitory effect of HFS on PC12 cells, instead of complete blockage.

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